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Rap-GEF Signaling Controls Stem Cell Anchoring to Their Niche through Regulating DE-Cadherin-Mediated Cell Adhesion in the *Drosophila* Testis

Hong Wang,[1,3] Shree Ram Singh,[1,3] Zhiyu Zheng,[1,3] Su-Wan Oh,[1] Xiu Chen,[1] Kevin Edwards,[2] and Steven X. Hou[1,*]

[1] The Laboratory of Immunobiology
National Institutes of Health National Cancer Institute at Frederick
Frederick, Maryland 21702
[2] Department of Biological Sciences
Illinois State University
Julian 210
Box 4120
Normal, Illinois 61790

Summary

Stem cells will undergo self-renewal to produce new stem cells if they are maintained in their niches. The regulatory mechanisms that recruit and maintain stem cells in their niches are not well understood. In *Drosophila* testes, a group of 12 nondividing somatic cells, called the hub, identifies the stem cell niche by producing the growth factor Unpaired (Upd). Here, we show that Rap-GEF/Rap signaling controls stem cell anchoring to the niche through regulating DE-cadherin-mediated cell adhesion. Loss of function of a *Drosophila* Rap-GEF (Gef26) results in loss of both germline and somatic stem cells. The *Gef26* mutation specifically impairs adherens junctions at the hub-stem cell interface, which results in the stem cells "drifting away" from the niche and losing stem cell identity. Thus, the Rap signaling/E-cadherin pathway may represent one mechanism that regulates polarized niche formation and stem cell anchoring.

Introduction

Stem cells can either self-renew or differentiate into short-lived cell types. Cancer cells also possess the potential for self-renewal; tumors may originate from a few transformed cancer stem cells (Reya et al., 2001). Understanding the molecular mechanisms that control stem cell self-renewal versus differentiation is crucial to the use of stem cells in regenerative medicine and the development of effective anticancer therapies. Accumulated evidence suggests that stem cells are controlled by particular microenvironments known as niches (Spradling et al., 2001; Fuchs et al., 2004). A niche is a subset of neighboring stromal cells and extracellular substrates. The stromal cells often secrete growth factors to regulate stem cell behavior.

The *Drosophila* testis provides an excellent in vivo system by which to study stem cells and niches at the cellular and molecular levels (Fuller, 1998; Lin, 2002; Gilboa and Lehmann, 2004; Yamashita et al., 2005). At the tip of the *Drosophila* testis (apex) is a germinal proliferation center, which contains the germline and somatic stem cells that maintain spermatogenesis (Figure 1B). Each adult male fly testis has 5–9 germline stem cells (GSCs). Each GSC is encysted by two somatic stem cells (SSCs, also called cyst progenitor cells). Both GSCs and SSCs attach to a group of 12 nondividing somatic cells, called the hub (Hardy et al., 1979; Lindsley and Tokuyasu, 1980; Gonczy and DiNardo, 1996). The hub defines the stem cell niche by expressing a growth factor, Unpaired (Upd), which activates the JAK/STAT pathway in GSCs to regulate the stem cell self-renewal (Kiger et al., 2001; Tulina and Matunis, 2001). Meanwhile, a member (*glass bottom boat* [*gbb*]) of the transforming growth factor (TGF-β) family is also expressed in the hub and plays a part in regulating GSC self-renewal by activating its corresponding signal transduction pathway in GSC (Kawase et al., 2003). Both Upd and Gbb are expressed in the hub and have very limited ability to diffuse; therefore, the GSCs first have to be anchored to the hub to receive the signals and maintain their stem cell identity. The cell adhesion molecules, DE-cadherin, encoded by the *shotgun* (*shg*), and β-catenin, encoded by *armadillo* (*arm*), are concentrated at the hub-GSC interface and may anchor the stem cells to the niche (Yamashita et al., 2003). However, how the adherens junctions are specifically formed at the hub-GSC interface is not clear. In this study, we demonstrate that Rap-GEF/Rap signaling specifically regulates adherens junction formation at the hub-GSC interface and may provide the extrinsic cue for the polarized niche formation.

Results

Gef26 Is Required for Germline Stem Cell Maintenance

In a large-scale screen for autosomal P element-induced zygotic lethal mutations (Oh et al., 2003; S.X.H. et al., unpublished data), we identified a semilethal mutation, *l(2)SH1450*. Escapers of the homozygous *l(2)SH1450* mutation have small, rough eyes and abnormal wings. Both mutant male and female flies are sterile. The P element *l(2)SH1450* was inserted into the 5′ promoter sequence of the *Gef26* gene, a PDZ domain guanine nucleotide exchange factor (GEF) for Rap GTPase (Figure 1A) (Lee et al., 2002). There are two previously reported *Gef26* alleles (Lee et al., 2002), *l(2)k13720* and *EP(2)0388*. We renamed *l(2)k13720* as $Gef26^1$, *EP(2)0388* as $Gef26^2$, and *l(2)SH1450* as $Gef26^3$. Through complementation tests, we identified an EMS allele of *Gef26*, $Gef26^4$, among our new EMS-induced lethal mutants. We also obtained two additional alleles of *Gef26*, $Gef26^5$ and $Gef26^6$, by imprecise mobilization of *l(2)SH1450*. $Gef26^4$ has a deletion of ~900 bp in the 5′ promoter sequence of the *Gef26* gene, $Gef26^5$ contains some residual P element sequences (~1.1 kb), and $Gef26^6$ has a 3 kb deletion surrounding the *l(2)SH1450* insertion site (Figure 1A).

In the male testis, the *Gef26* mRNA expressions in $Gef26^3/Gef26^4$, $Gef26^4/Gef26^5$, and $Gef26^4/Gef26^6$ are

*Correspondence: shou@mail.ncifcrf.gov
[3] These authors contributed equally to this work.

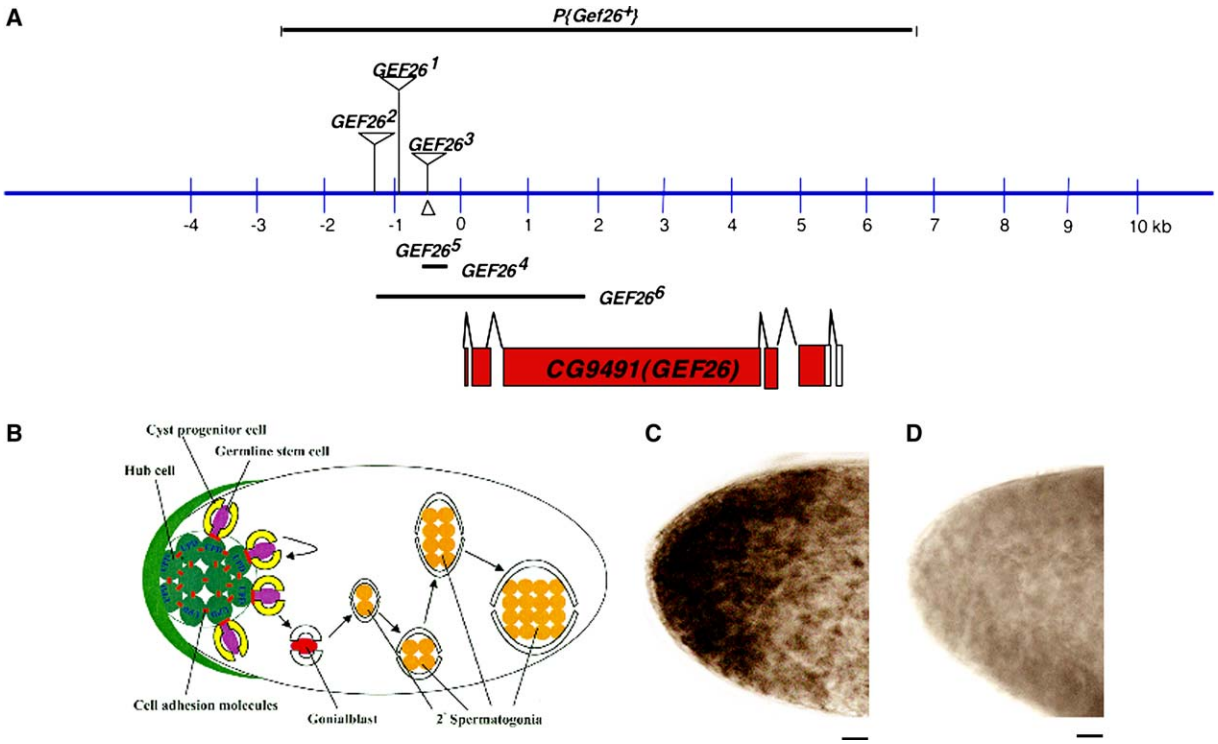

Figure 1. Analysis of *Gef26* Mutants

(A) *Gef26* exon sequences are indicated by boxes. The coding sequence is indicated by filled boxes; the 3′ untranslated region is represented by open boxes. Breaks between the boxes indicate introns. Triangles indicate insertion sites of P elements. The sizes and positions of the deletions in $Gef26^4$ and $Gef26^6$ are indicated by the thick, black lines. The black bar at the top indicates the DNA fragment used in *p{Gef26+}* for genomic rescue.

(B) A sagittal section of the *Drosophila* testis apex is drawn schematically and leaves out most of the cells for clarity. Both GSCs (pink) and SSCs (yellow) are anchored around the hub (green) through adherens junctions. Asymmetric division of a stem cell results in spermatogenic cysts, in which each gonialblast (red) is encased by two somatic cyst cells (white). Four more consecutive divisions produce a cyst of 16 spermatogonia.

(C and D) *Gef26* mRNA expressions in the (C) wild-type and (D) mutant male testis. Scale bars in (C) and (D) represent 10 μm.

undetectable (Figure 1D; data not shown) by in situ hybridization; they may correspond to either a *Gef26* null or strong loss-of-function condition. $Gef26^1$ and $Gef26^2$ are hypomorphic alleles.

Both male and female flies of $Gef26^3/Gef26^4$, $Gef26^4/Gef26^5$, and $Gef26^4/Gef26^6$ are sterile. We examined the ovaries of the mutant females and found that the mutant flies have various oogenesis defects, but normal germline stem cells (data not shown). To determine whether there are any defects in the stem cells in mutant male testes, we stained testes with anti-Fas III (to mark the hub cells), mAb1B1 (to mark the fusome), and anti-Vasa (to mark the germ cells) antibodies. While the hub and 7–9 GSCs (an average of 8.2 GSCs, n = 53) can be clearly visualized in wild-type testes (Figure 2A), the GSCs in *Gef26* mutant testes are either dramatically reduced (Figure 2B; an average of 1.8 GSCs, n = 34) or completely lost (Figures 2C and 2E; an average of 0.7 GSCs, n = 45). Fas III stainings are also absent in most of the mutant testes (Figures 2C and 2E). The 2° spermatogonia with branching fusomes (Figures 2C and 2E) moved to the tip.

When an 11.6 kb genomic rescue construct encompassing the *Gef26* gene only (*p{Gef26+}*) (Figure 1A) was introduced into *Gef26* mutant male flies, the stem cells and Fas III staining were recovered (Figures 2D and 2F). Therefore, stem cell loss is due to *Gef26* mutation.

To further verify the stem cell loss phenotype of *Gef26* mutations, we examined the expression of the STAT92E protein and M5-4 marker. JAK/STAT signaling is required in GSCs for GSC self-renewal (Kiger et al., 2001; Tulina and Matunis, 2001). In the wild-type testis, STAT92E protein was detected specifically in GSCs and gonialblasts (Figure 3A). In *Gef26* mutant testes, STAT92E expression was almost completely lost (Figure 3B). The enhancer trap line M5-4 drives β-galactosidase expression in hub cells, GSCs, and gonialblasts (Tran et al., 2000) (Figure 3C) in wild-type testes. In all *Gef26* mutant testes examined, the number of β-galactosidase-positive cells decreased dramatically (Figure 3D). However, the expression of β-galactosidase and Arm (Figure 3D) in the hub looks normal in the *Gef26* mutant testis, suggesting that Gef26 does not regulate general hub formation and only affects the expression of individual gene (such as Fas III; Figures 2C and 2E) in the hub.

We next examined the cytoplasmic form of Bam protein (BamC), which is expressed in 2-cell to early 16-cell cysts (Kiger et al., 2000; Tran et al., 2000) (Figure 3E). We used a *bam-GFP* transgene (a *bam* promoter fused to the GFP gene) to examine *bam* gene expression (Chen and McKearin, 2003; Kawase et al., 2003). Consistent with earlier reports, we found that *bam* was expressed in 2- to 16-cell spermatogonia, but not in GSCs and gonialblasts in the wild-type testis (Figure 3E). In the wild-type

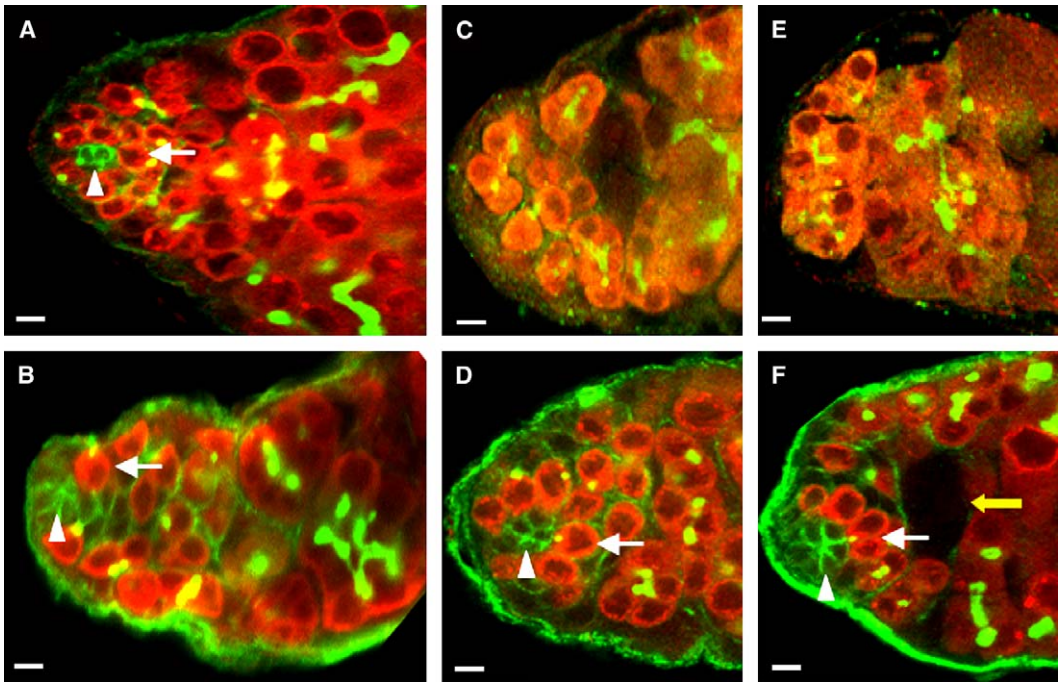

Figure 2. Gef26 Is Required for GSC Maintenance

(A–F) Testes immunostained with anti-vasa antibody to label germ cells (red), anti-Fas III antibody to label the hub (green, arrowheads), and mAb1B1 (green) to label fusomes. (A) Wild-type control testis. GSCs (arrow) contact the Fas III-positive hub (arrowhead). (B) *Gef26*^{3}/*Gef26*^{4} testis. Only two GSCs remain. (C) *Gef26*^{4}/*Gef26*^{5} testis. No Fas III staining or GSCs are visible, and spermatogonia with branched fusomes move to the tip. (D) A transgene expressing *p{Gef26+}* rescues the *Gef26*^{4}/*Gef26*^{5} male testis to normal. The Fas III-positive hub (green, arrowhead) and GSCs with unbranched fusomes (arrow) are recovered. (E) *Gef26*^{4}/*Gef26*^{6} testis. No Fas III staining or GCSs are visible, and spermatogonia with branched fusomes move to the tip. (F) The *p{Gef26+}* transgene significantly rescues the *Gef26*^{4}/*Gef26*^{6} testis phenotype. The Fas III-positive hub (green, arrowhead) and GSCs with unbranched fusomes (arrow) are recovered. However, some spermatogonia are still missing (yellow arrow). Scale bars represent 10 μm.

testis, the GSCs and gonialblasts comprise the GFP-negative cells between the hub and the first rows of spermatogonia (Figure 3E, line), as illustrated by unbranched fusomes. In the *Gef26* mutant testis, GFP-positive 2° spermatogonia and cells with branched fusomes moved to the tip, and GSCs and gonialblasts with unbranched fusomes were lost (Figure 3F).

Gef26 Is Required for Somatic Stem Cell Maintenance

Gef26 activity was also required for the maintenance of SSCs in the testis. In the wild-type testis, each GSC is encysted by two SSCs. Both GSCs and SSCs attach to the hub cells (Hardy et al., 1979; Lindsley and Tokuyasu, 1980; Gonczy and DiNardo, 1996) (Figure 1B). The SSCs self-renew and give rise to the somatic cyst cells that enclose the gonialblasts and spermatogonia (Hardy et al., 1979; Lindsley and Tokuyasu, 1980; Gonczy and DiNardo, 1996) (Figure 1B). To verify the SSC change in *Gef26* mutant testes, we examined expression of the Traffic jam protein (Tj) and enhancer trap line 842 marker. Tj is a transcription factor and is expressed in the SSCs and early cyst cells in wild-type testes (Kiger et al., 2001) (Figure 4A). In *Gef26* mutant testes, Tj expression was significantly reduced (Figure 4B). The enhancer trap line 842 drives β-galactosidase expression in hub cells, SSCs, and cyst cells (Fabrizio et al., 2003) (Figure 4C) in wild-type testes. In all *Gef26* mutant testes examined, the number of β-galactosidase-positive cells decreased dramatically (Figure 4D).

Stem cell loss might be caused by cell death. We examined cell death in wild-type and *Gef26* mutant testes by using an Apop Tag kit. No dying GSCs were detected in the wild-type testis, and some dying spermatogonial cysts were detected (Figure 4E, white arrow), as previously reported (Kawase et al., 2003; Brawley and Matunis, 2004). In the *Gef26* mutant testis, an increased number of dying cell clusters were detected in late-stage cysts, but not in GSCs and gonialblasts (Figure 4F, white arrows). Thus, differentiation accounts for the loss of GSCs and SSCs in *Gef26* mutant testes.

The Rap-GEF/Rap Signal Transduction Pathway Regulates Stem Cell Maintenance

Because Gef26 is a guanine nucleotide exchange factor for Rap GTPase, we next examined the genetic interaction between *Gef26* and *Rap*. There are two *Rap* genes in the *Drosophila* genome; they were named *Rap1* and *Rap2l* (Ras-associated protein 2-like). We used the *Rap1*$^{rv(R)B1}$, a possible null allele (Asha et al., 1999), and the *Rap2l*^{1} allele, a P element inserted immediately downstream of the ATG translation starting code (Oh et al., 2003). The testis of the weak allele combination of *Gef26*^{1}/*Gef26*^{3} has normal GSCs and STAT92E protein expression (Figure 5A and Figure S1A; see the Supplemental Data available with this article online); the

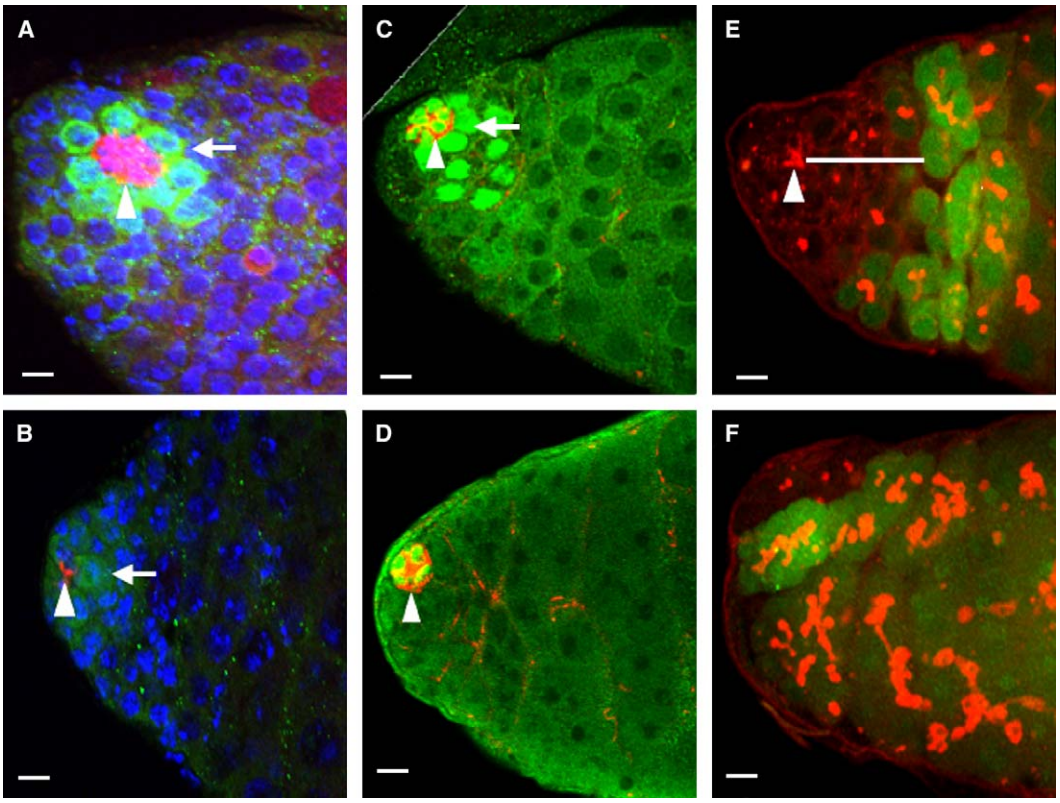

Figure 3. Gef26 Regulates JAK/STAT Signaling and GSC Fate

(A and B) Testes immunostained with anti-STAT92E antibody (green), anti-Fas III antibody (red), and DAPI (blue). (A) Wild-type control testis. STAT92E is specifically expressed in GSCs and weakly expressed in gonialblasts (arrow). (B) In the *Gef26*^{3}/*Gef26*^{4} testis, only a very small amount of Fas III remains, and STAT92E expression is dramatically reduced.

(C and D) Testes immunostained with anti-β-galactosidase (green) from the M5-4 marker and anti-Arm (red, arrowhead). (C) Wild-type testis with the M5-4 marker. β-galactosidase (green) is expressed in the hub (arrowhead), GSCs (arrow), and nearby gonialblasts. (D) In the *Gef26*^{3} M5-4/*Gef26*^{4} testis, β-galactosidase stainings are essentially lost in GSCs and gonialblasts, but are expressed in the hub.

(E and F) Testes immunostained with anti-GFP (green), anti-Fas III (red, arrowhead), and mAb1B1 (red). (E) Wild-type testis with Bam-GFP marker. GFP is expressed in the spermatogonia. The arrowhead points to the Fas III-positive hub; the line highlights the distance between the hub and GFP-positive spermatogonia. (F) In the *Gef26*^{3} Bam-GFP/*Gef26*^{4} testis, no Fas III staining or GSCs are visible, and GFP-positive spermatogonia with branched fusomes move to the tip.

Scale bars represent 10 μm.

number of GSCs and STAT92E expressions was significantly reduced once one copy of Rap1 (Figure 5B and Figure S1B) or Rap2l (Figure S1C) was removed from the *Gef26*^{1}/*Gef26*^{3} background. Thus, reduction of either Rap1 or Rap2l activity enhances the stem cell loss phenotype of the *Gef26* mutation, and the Rap-GEF/Rap pathway regulates stem cell maintenance.

The EGF Receptor, JAK/STAT, and Rap-GEF/Rap Pathways Cooperatively Regulate Stem Cell Fates

Recent works have demonstrated that two extrinsic signals regulate GSC self-renewal or differentiation. The hub defines the stem cell niche by producing a growth factor, Upd, and Upd then activates the JAK/STAT pathway in GSCs to regulate the cell self-renewal (Kiger et al., 2001; Tulina and Matunis, 2001). Meanwhile, activation of the EGF receptor/Ras/Raf/MAPK pathway produces an unidentified signal in the SSCs that limits self-renewal and promotes differentiation of GSCs (Kiger et al., 2000; Tran et al., 2000). To examine the potential genetic interaction between Gef26 and the JAK/STAT signal transduction pathway, we overexpressed *upd* in the germline in the *Gef26* mutant background (Figure 5C). Forced germline expression of *upd* in both wild-type and *Gef26* mutant testes results in similar phenotypes: the testes are greatly enlarged and filled with many small GSCs with unbranched fusomes (Kiger et al., 2001; Tulina and Matunis, 2001) (Figure 5C). Most ectopic GSCs are not attached to the hub, indicating that uncontrolled stem cell proliferation and self-renewal occur independent of contact with the hub. These data suggest that the JAK/STAT signal transduction pathway functions downstream of Gef26 in regulating stem cell proliferation and self-renewal. Our data, described in the later sections, suggest that Gef26 controls the attachment of GSCs to the hub through regulating the adherens junctions at the hub-GSC interface; thus, it makes sense that the ectopic GSCs in testes overexpressing *upd* are independent of the hub and the Gef26 function.

We further examined the genetic interaction between Gef26 and EGF receptor signaling. We tested whether the stem cell loss phenotype of *Gef26*^{3}/*Gef26*^{4} male flies could be alternated when the gene dose of EGF receptor (*Egfr*) was reduced (Figure 5D). The stem cell loss phenotype of *Gef26*^{3}/*Gef26*^{4} could be significantly rescued when one gene dose of *Egfr* was eliminated

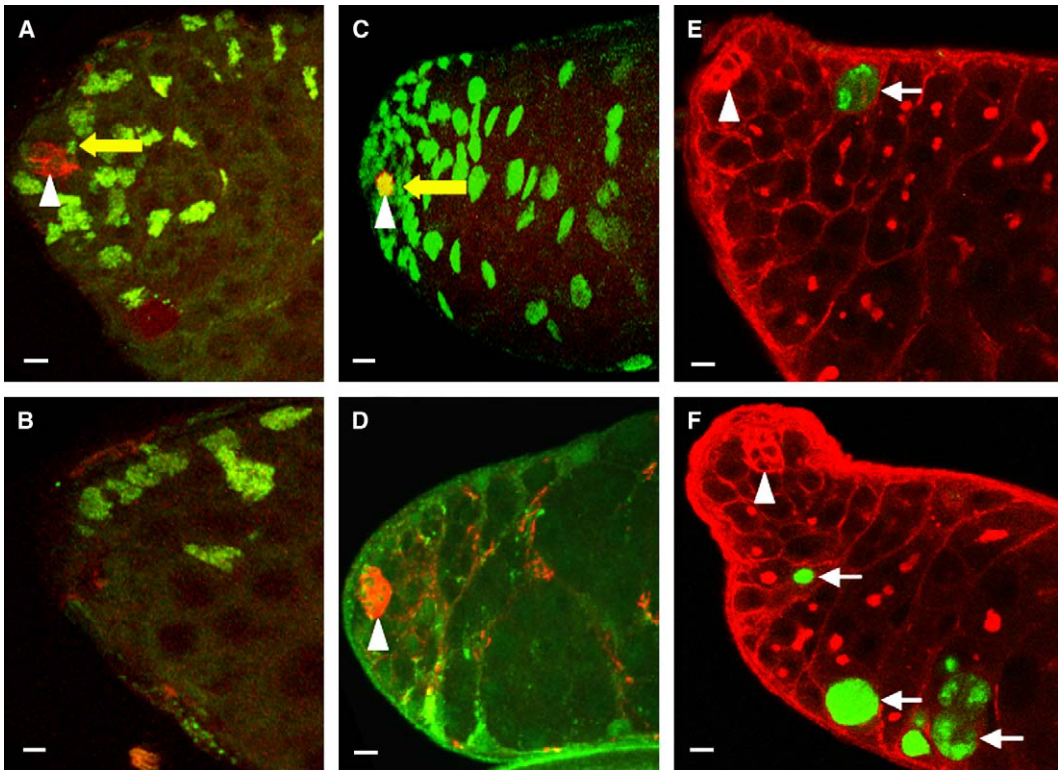

Figure 4. Gef26 Is Required for SSC Maintenance

(A and B) Testes immunostained with anti-Tj (green) and anti-Fas III (red). (A) Wild-type testis. Tj is expressed in SSCs (yellow arrow) and early cyst cells. (B) In the *Gef26*^{3}/*Gef26*^{4} testis, both Tj and Fas III stainings are dramatically reduced.

(C and D) Testes immunostained with anti-β-galactosidase (green) from the 842 marker and anti-Arm (red). (C) Wild-type testis with the 842 marker. β-galactosidase (green) is expressed in the hub, SSCs (yellow arrow), and early cyst cells. (D) In the *Gef26*^{3} 842/*Gef26*^{4} testis, β-galactosidase staining is essentially lost from SSCs and early cyst cells, but the β-galactosidase staining and Arm stainings in the hub look normal.

(E and F) Testes were stained to detect dying cells (green). (E) In the wild-type testis, few dying spermatogonial cysts were detected (green, arrow). (F) In the *Gef26*^{4}/*Gef26*^{6} testis, increased dying cell clusters in later cysts were detected (green, arrows). Arm staining in the hub (red, arrowhead) looks normal.

Scale bars represent 10 μm.

(Figure 5D, compare with Figure 3B). The *Gef26*^{3}/*Gef26*^{4} fly testis is *Gef26* null based on in situ hybridization data (Figure 1D), and the EGF receptor likely modifies the stem cell loss phenotype of *Gef26* mutation through a pathway either downstream of or parallel to Gef26.

The Gef26 Function Is Not Required in the Germline for Stem Cell Maintenance

To determine whether Gef26 function is required in the germline or in the surrounding somatic cells for stem cell maintenance, we generated both wild-type and *Gef26* mutant clones in GSCs (Figure 5E; data not shown). Seven and 11 days after clone induction, compatible numbers of marked wild-type and *Gef26* mutant GSCs were detected. Seven days after clone induction, one or more marked *Gef26* mutant GSC was found in 74% of testes (23/31) in both *Gef26*^{3} and *Gef26*^{6} mutant clones (Figure 5E; data not shown), whereas 11 days after clone induction, 61% (17/28) of the testes still carried one or more marked *Gef26* mutant GSC (data not shown). In all cases, progression through spermatogenesis is normal, as judged by normal cysts with 16 differentiating spermatocytes (Figure 5E). Thus, *Gef26* function is not required in the germ cells.

GSCs "Drifted away" from the Niche and Lost Stem Cell Identity in *Gef26* Mutant Testes

To understand the molecular mechanism of Gef26-regulating GSCs, we examined GSC formation in third instar larvae by using the M5-4 marker, which labels hub cells, GSCs, and gonialblasts (Tran et al., 2000). While the marker is expressed as a tight cluster around the hub in the testes of wild-type larvae (Figure 6A), a number of marked cells drifted away from the hub in the testes of *Gef26* mutant larvae (Figure 6B). In *Gef26* mutant adult testes, most germ cells also drifted away from the hub (Figures 6C and 6D); in a few instances, we detected one GSC still attached to the hub through DE-cadherin-positive adherens junctions (Figure 6D, arrow). These data suggest that stem cells in the *Gef26* mutant testes drifted away from the niche and lost their stem cell status due to impaired anchoring.

Gef26 Regulates the Formation of Adherens Junctions at the Hub-GSC Interface

We further investigated the connection between Gef26 and adherens junctions. As previously reported (Yamashita et al., 2003), high levels of DE-cadherin and Arm are expressed at the hub-GSC interface as well as at

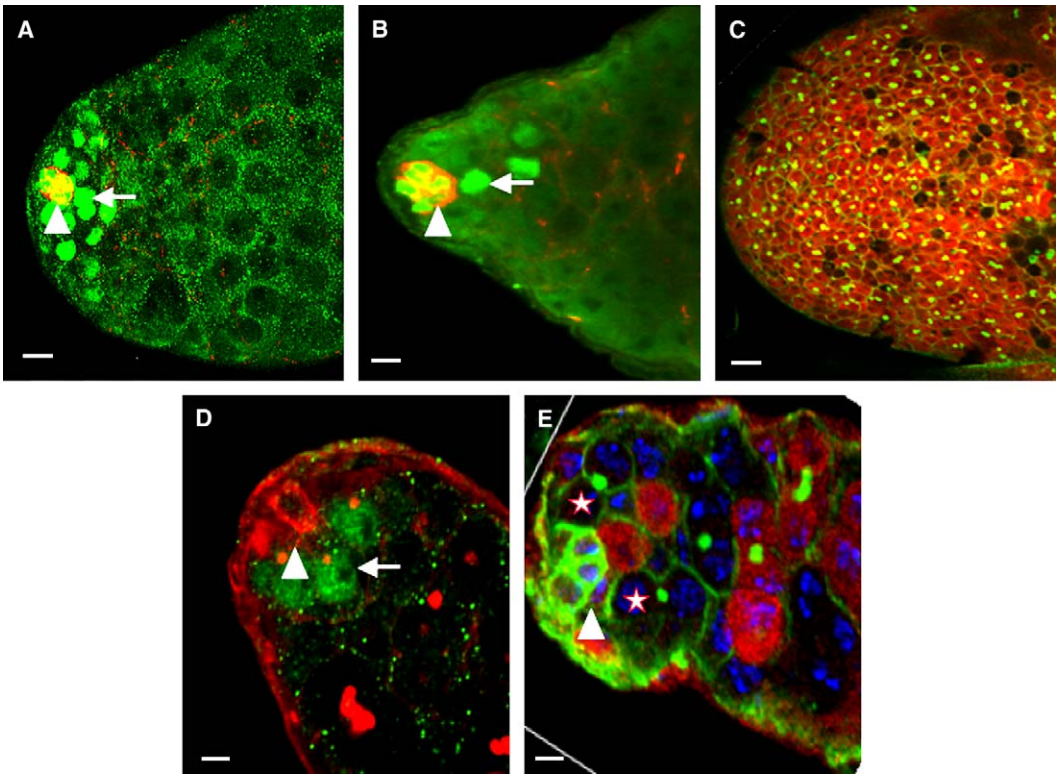

Figure 5. The EGF Receptor, JAK/STAT, and Rap-GEF/Rap Pathways Cooperatively Regulate Stem Cell Fates

(A and B) Testes immunostained with anti-β-galactosidase (green) from the M5-4 marker and anti-Arm (red). The testis of the weak allele combination of *Gef26*^{1}/*Gef26*^{3} has normal GSCs (arrow, compare with Figure 3C). (B) The expressions of β-galactosidase and GSCs (green, arrow) were significantly reduced once one copy of Rap1 was removed in the *Gef26*^{1}/*Gef26*^{3} background.

(C) *UAS-upd Gef26*^{3}/*Gef26*^{4} ; *nos-Gal4*/+ testis. Ectopic GSC-gonialblast pairs with spherical fusomes are dramatically expanded. Most ectopic GSC-gonialblast pairs are not attached to the hub.

(D) *Gef26*^{3} *Egfr*^{f24}/*Gef26*^{4} testis. The hub and GSCs are revealed by anti-Fas III (red, arrowhead) and anti-STAT92E (green, arrow). Reduction of *Egfr* activity significantly rescues the *Gef26*^{3}/*Gef26*^{4} phenotype (compare [D] with Figure 3B).

(E) Confocal section through the apex of the testis containing *Gef26*^{6} clones at day 7. The testis is immunostained with anti-β-galactosidase (red), anti-Fas III (green, arrowhead), and mAb1B1 (green). Two *Gef26* null GSC clones (stars) and a number of *Gef26* null spermatogonia clones that are β-galactosidase negative are found in this testis.

Scale bars represent 10 μm.

the interface between the hub cells in wild-type testes (Figures 6E and 6G). In *Gef26* mutant testes, DE-cadherin expression (and, to a lesser degree, Arm expression) at the hub-GSC interface is significantly reduced (Figures 6F and 6H, arrows). To verify this phenotype, we examined the expression of another component of adherens junctions, *Drosophila* α-catenin (*Dα-catenin*). We specifically expressed a *UAS-Dα-catenin-GFP* transgene in the hub (Figure 6I). In the *Gef26* mutant testis, the GFP expression at the hub-GSC interface is significantly reduced, while the GFP expression inside the hub looks normal (Figure 6J, arrow).

Hub Rap-GEF/Rap Signaling Regulates Adherens Junctions and Anchoring of Stem Cells to Their Niches

To investigate the origin of the Rap-GEF/Rap signaling that regulates the formation of adherens junctions at the hub-GSC interface, we first examined the expressions of Gef26 and Rap1 proteins. We used an anti-Gef26 polyclonal antibody to detect Gef26 protein (see Experimental Procedures). To detect Rap1 protein, we used a transgene encoding a GFP-Rap1 fusion protein (Knox and Brown, 2002). This fusion protein is driven by the endogenous *Rap1* promoter. In wild-type testes, both Gef26 (Figures 7A and 7C) and GFP-Rap1 (Figure S1D) were highly concentrated at the hub-GSC interface and between the hub cells. The expression of Gef26 and Rap-GFP in the hub suggests that the signal is from the hub.

Further, forced expression of a constitutively activated form of Rap1 (*Rap1*^{V12}) (Boettner et al., 2003), specifically in the hub in *Gef26* mutant testes (Figures 7D and 7E), rescued the adherens junctions and GSCs to wild-type. However, overexpression of DE-cadherin either in the hub or in early germ cells (Figures 7F and 7G; data not shown) rescued the adherens junctions and GSCs to wild-type, suggesting that overexpression of DE-cadherin in either the hub or GSCs may strengthen the adherens junctions at the hub-GSC interface and rescue the *Gef26* mutant phenotype. We also tried, but failed, to generate *shg* null GSC clones after examining a large number of testes (data not shown), suggesting that *shg* may be necessary for viability or proliferation

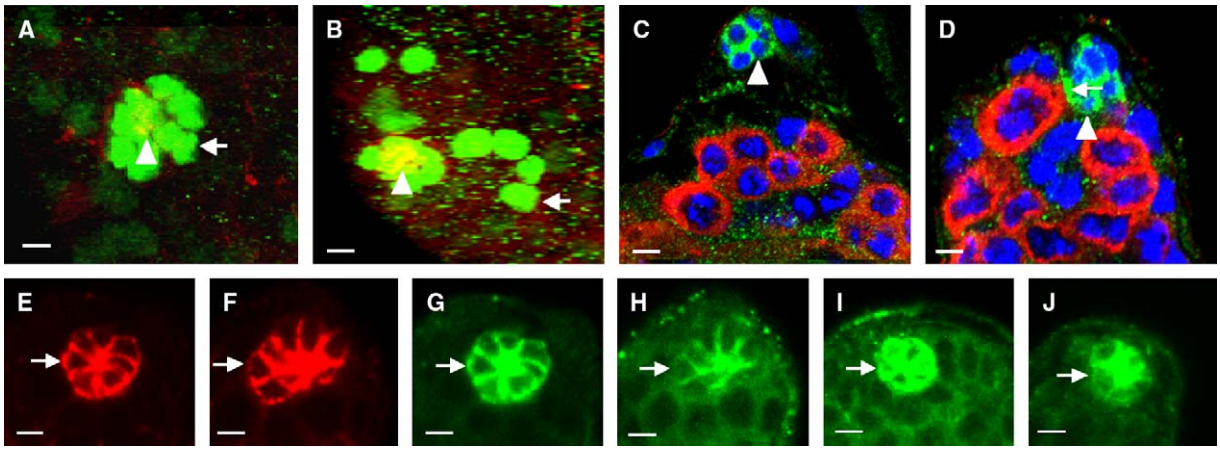

Figure 6. GSCs "Drift away" from the Niche in *Gef26* Mutant Testes Due to Impaired Adherens Junctions

(A and B) Third instar larvae testes immunostained with anti-β-galactosidase (green) from the M5-4 marker and anti-DE-cadherin (red, arrowheads). (A) In the wild-type larvae testis, the M5-4 marker (green, arrow) is expressed in the hub (arrowhead), GSCs, and nearby gonialblasts (arrow). (B) In the $Gef26^{3}$ M5-4/$Gef26^{4}$ larvae testis, some β-galactosidase-positive cells drift away from the hub (arrow).

(C and D) Two testes of $Gef26^{4}$/$Gef26^{6}$ adult flies immunostained with anti-DE-cadherin (green), anti-Vasa (red), and DAPI (blue). In (C), all Vasa-positive germ cells drifted away from the hub (arrowhead); in (D), only one GSC is still attached to the hub through DE-cadherin-positive adherens junctions (arrow).

(E–H) Testes immunostained with (E and F) anti-Arm or with (G and H) anti-DE-cadherin. (E) and (G) are wild-type testes; (F) and (H) are $Gef26^{3}$/$Gef26^{4}$ testes. (E and G) In wild-type testes, high levels of Arm and DE-cadherin are expressed at the hub-GSC interface (arrows) as well as the interfaces between the hub cells. (F and H) In *Gfe26* mutant testes, DE-cadherin (and, to a lesser degree, Arm) expression at the hub-GSC interface is significantly reduced (arrows).

(I and J) Testes immunostained with anti-GFP antibody. (I) The *upd-Gal4/Y; UAS-Dα-catenin-GFP/+* testis. GFP is expressed in the hub and at the hub-GSC interface (arrow). (J) The *upd-Gal4/Y;* $Gef26^{3}$ *UAS-α-Dcatenin-GFP/*$Gef26^{4}$ testis. While GFP expression inside the hub looks normal, GFP expression at the hub-GSC interface is significantly reduced (arrow).

Scale bars in (A)–(D) represent 5 μm. Scale bars in (E)–(J) represent 3 μm.

of GSCs. These data suggest that hub Rap-GEF/Rap signaling regulates adherens junctions at the hub-GSC interface to anchor the GSCs to their niche.

Discussion

Rap-GEF/Rap Signaling Regulates the Formation of Adherens Junctions

Rap1 was first identified as a gene that can reverse the transformed phenotype of fibroblasts by one of the mutated Ras genes, *K-ras* (Kitayama et al., 1989). It belongs to the Ras family of small GTP binding proteins. Its apparent tumor suppressor properties were initially proposed to antagonize the activity of Ras by competing for a common target (or regulatory protein). However, recent studies have suggested that Rap1 may actually regulate adherens junctions. Cell-cell junctions are formed evenly around the lateral circumference of cells by homophilic interactions between the extracellular domains of E-cadherin, linked by their intracellular tail to catenins and to the actin cytoskeleton (Jamora and Fuchs, 2002). Two recent studies in mammals have shed new lights on the connection between Rap1 and adherens junctions. In the first study, a Rap1 GTPase activator, DOCK4, was identified as a tumor suppressor (Yajnik et al., 2003). DOCK4 specifically activates Rap1 and regulates the formation of adherens junctions. In the second study, the authors demonstrated that ligation of the extracellular domain of E-cadherin enhances Rap1 activity, and that active Rap1 regulates the subsequent accumulation of E-cadherin at newly formed cell-cell contact sites (Hogan et al., 2004). Data from the second study suggest that formation of adherens junctions is a two-step process. When cells first contact one another, small clusters of E-cadherin ligate through the homophilic interaction, which may, in turn, induce the activation of Rap1; activation of Rap1 may then activate inside-to-outside signaling through stimulating actin polymerization, which mediates the further recruitment of E-cadherin from the cytoplasmic or plasma membrane pool and facilitates the formation of mature E-cadherin-based adherens junctions.

In *Drosophila*, a previous study reported that Rap1 regulates the even distribution of adherens junctions of epithelial cells in wing imaginal disc (Knox and Brown, 2002); Rap1 null cells have uneven adherens junctions and are dispersed into neighboring cells. In this study, we demonstrated that Rap-GEF/Rap signaling controls the adherens junction formation at the hub-GSC interface in fly testes. Disruption of signaling impairs the adherens junctions at the hub-GSC interface and causes stem cells to drift away from the hub and lose stem cell identity.

Formation of Asymmetric Stem Cell Division in Fly Testes

In this study, we demonstrated that Rap-GEF/Rap signaling regulates the adherens junction formation at the hub-GSC interface. A previous study (Yamashita et al., 2003) showed that the tumor suppressor APC homologs and the integral centrosome component centrosomin (CNN) may interact with the DE-cadherin-mediated adherens junctions at the hub-GSC interface to orient mitotic spindles of GSCs perpendicular to the hub for

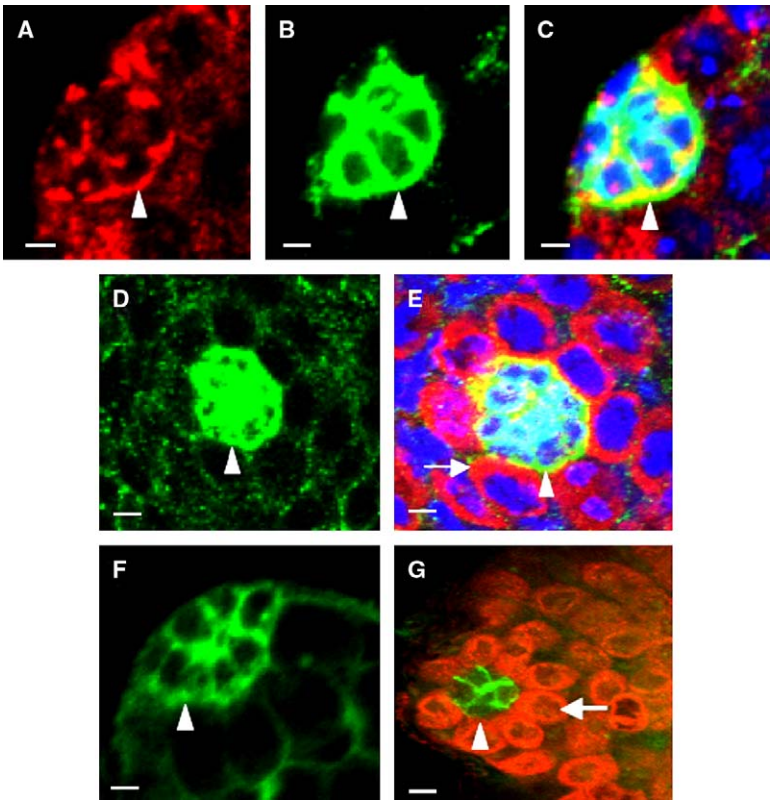

Figure 7. Hub Rap-GEF/Rap Signaling Regulates the Formation of Adherens Junctions at the Hub-GSC Interface

(A and B) Wild-type testes were immunostained with (A) anti-Gef26 (red) or with (B) anti-Arm (green).

(C) A merge of (A) and (B). Gef26 protein is mainly expressed at the hub-GSC interface (arrowheads in [A]–[C]).

(D and E) *upd-Gal4/Y;Gef26*^{4} */Gef26*^{6} ; *UAS-Rap1*^{V12}/+ testes. Expression of a constitutively activated form of Rap1 in the hub rescues DE-cadherin expression at the hub-GSC interface ([D], arrowheads) and GSCs ([E], arrow).

(F and G) The *upd-Gal4/Y;Gef26*^{3} *UAS-DEFL* / *Gef26*^{4} testes. Forced expression of DE-cadherin specifically in the hub rescues DE-cadherin expression at the hub-GSC interface ([F], arrowhead) and GSCs ([G], arrow). The Fas III staining (green, arrowhead) and the Vasa-positive GSCs (arrow) are recovered in (G).

Scale bars in (A)–(F) represent 3 μm. The scale bar in (G) represents 10 μm.

asymmetric stem cell division in the fly testis (Yamashita et al., 2003).

Mechanisms that regulate the formation of asymmetric adherens junctions may be evolutionally conserved. In budding yeast, a cortical landmark formed at the site of cytokinesis aligns the spindles of mother and daughter cells. The Rap GTPase module is required for budding and recruits factors for the polarized organization of the actin cytoskeleton and cell-cell or cell-matrix interactions (Cabib et al., 1998; Arkowitz, 2001; Kang et al., 2001; Marston et al., 2001). The module consists of the Rap-like GTPase, Bud1/Rsr1; its GEF, Bud5; and its GTPase activating protein (GAP), Bud2. The selection of a specific budding site is initiated by Bud5 localization at the cell division site through its physical interaction with a transmembrane protein, Bud10/Axl2 (Kang et al., 2001; Marston et al., 2001). Axl2 possesses domains similar to those of integrins. The budding complex further controls microtubule spindle orientation through Kar9 (a possible adenomatous polyposis coli [APC] homolog) (Miller and Rose, 1998). An evolutionarily conserved Rap-GEF-Rap/APC pathway may control both budding site selection in yeast and the asymmetric stem cell division in the fly testis. Bud5 is specifically expressed at the budding site, and its expression determines the cell polarization pattern. In fly testes, both Gef26 (Figures 7A and 7C) and GFP-Rap1 (Figure S1D) were specifically expressed at the hub-GSC interface and between the hub cells; whether Gef26 expression determines GSC polarization pattern remains to be explored.

Based on our current results and earlier studies, we propose that the interaction between the hub and GSCs is a three-step process. First, Rap-GEF/Rap-mediated signaling from the hub may guide the asymmetric formation of adherens junctions at the hub-GSC interface. Second, astral microtubules in GSCs are anchored to the adherens junctions through APCs, and the GSCs are polarized. Third, after a proper position has been established between the hub and GSCs, the signal transduction pathways, such as the JAK/STAT, the EGF receptor/Ras/Raf, and Dpp pathways, regulate either GSC self-renewal or GSC differentiation. Similar processes may be involved in stem cell regulation in other organisms. Our findings should provide general guiding principles for stem cell research.

Experimental Procedures

Drosophila Stocks

Oregon R was used as wild-type. *Gef26*^{1} (*l(2)k13720*) and *Gef26*^{2} (*EP(2)0388*) were previously described (Lee et al., 2002). *l(2)k13720* was obtained from the Bloomington stock center, and *EP(2)0388* was obtained from the Szeged stock center. *Gef26*^{3} (*l(2)SH1450*) and *Rap2l*^{l} (*l(2)SH0581*) were isolated in a P element-induced mutagenesis screen (Oh et al., 2003; S.X.H. et al., unpublished data). *Gef26*^{4} was isolated in an EMS-induced mutagenesis screen (S.X.H. et al., unpublished data). *Gef26*^{5} and *Gef26*^{6} were generated after imprecise excisions of *l(2)SH1450*.

The other following fly stocks used in this study were described either in FlyBase or are as otherwise specified: *Rap1*^{B1} (provided by I. Hariharan) (Asha et al., 1999); 842 is a P element enhancer trap that expresses LacZ in the hub, SSCs, and cyst cells of the testis (provided by S. DiNardo) (Fabrizio et al., 2003); M5-4 is a P element enhancer trap that expresses LacZ in the hub, GSCs, and gonialblasts (provided by S. DiNardo) (Tran et al., 2000; Fabrizio et al., 2003); *bam-GFP* (provided by T. Xie) (Chen and McKearin, 2003); *UAS-DEFL* (full-length *shg*) #6-3 (Oda and Tsukita, 1999b) and *UAS-DαC-GFP #3* (Dα-catenin tagged with GFP) (Oda and Tsukita, 1999a)

were obtained from the Kyoto stock center; *Rap1-GFP* (provided by N. Brown) (Knox and Brown, 2002); *upd-Gal4* (provided by T. Xie) (Kawase et al., 2003); *UAS-upd* (Chen et al., 2002); *UAS-Rap1*^{V12} (provided by U. Gaul) (Boettner et al., 2003); *FRT*^{42D}-*shg*^{R69} (provided by U. Tepass); *yw hs-FLP; FRT*^{40A} *arm-lacZ* and *yw hs-FLP; FRT*^{42D} *arm-lacZ* were provided by T. Xie; *nos-Gal4* (nanos-Gal4VP16) (Van Doren et al., 1998) and *Egfr*^{f24} (*Egfr*^{CO}) were obtained from the Bloomington stock center.

Flies were raised on standard *Drosophila* media at 25ºC unless otherwise indicated. Chromosomes and mutations that are not described in the text can be found in FlyBase.

Generating Mutant GSC Clones

Clones of mutant GSCs were generated as previously described (Kawase et al., 2003). To generate *Gef26* mutant GSC clones, *FRT*^{40A} *+, FRT*^{40A} *Gef26*^{3}*/Cyo* and *FRT*^{40A} *Gef26*^{6}*/Cyo* males were mated with virgin females of genotype *yw hs-FLP; FRT*^{40A} *arm-lacZ*. To generate *shg* mutant GSC clones, *FRT*^{42D} + or *FRT*^{42D} *shg*^{R69}*/Cyo* males were mated with virgin females of genotype *yw hs-FLP; FRT*^{42D} *arm-lacZ*. One- or 2-day-old adult males carrying an *arm-lacZ* transgene in *trans* to the mutant-bearing chromosome were heat shocked six times at 37ºC for 1 hr, separated by 8–12 hr. The males were transferred to fresh food every day at 25ºC. The testes were removed at 2, 5, or 7 days after the first heat-shock treatment and were then processed for antibody staining.

In Situ Hybridization

In situ hybridizations to whole-mount testes by using a digoxigenin-labeled anti-sense *Gef26* RNA probe were performed as described (Hou et al., 1996).

Immunofluorescence Staining and Microscopy

Testes were dissected in Ringer's and fixed for 40 min in 4% formaldehyde-PBX (PBS plus 0.1% Triton X-100). After several washes with PBX, the samples were preabsorbed overnight at 4ºC in PBX-2 (PBX plus 2% normal goat serum). Testes were rinsed once with PBX and then incubated overnight at 4ºC in primary antibody diluted in PBX containing 0.5% BSA. After several washes with PBX, testes were incubated with secondary antibody diluted in PBX containing 0.5% BSA for 2 hr at room temperature. After several more washes with PBX, testes were first treated with 0.04 mg/ml RNase A for 30 min and then incubated in 1 μg/ml DAPI for 5 min. After several more washes, the tissues were mounted in a PBS:glycerol (1:1) solution. Confocal images were obtained by using a Zeiss LSM510 system, and were processed with Adobe Photoshop 7.0.

The following antisera were used: rabbit polyclonal anti-STAT92E antibody (1:500) (Chen et al., 2002), rabbit polyclonal anti-Vasa antibody (1:5000; gift from R. Lehmann), rabbit polyclonal anti-β-Gal antibody (1:1000; Cappel), mouse monoclonal anti-Hts antibody 1B1 (1:4; Developmental Studies Hybridoma Bank [DSHB]), rat monoclonal anti-DE-cadherin antibody (1:100; gift from T. Uemura), mouse monoclonal anti-Armadillo N7A1 (1:4; DSHB), mouse monoclonal anti-Fas III antibody (1:10; DSHB), rabbit polyclonal anti-GFP antibody (1:200; Molecular Probes), rat polyclonal anti-Traffic jam (Tj) (1:500; gift from D. Godt). A peptide corresponding to peptides 1548–1567 (amino acid sequence: GKTTGPQERWFPDCRPTTKQ) of Gef26 was used to produce antibodies in rabbits. Antiserum was purified by using the peptides as affinity reagents. Gef26 staining was performed by using the purified antisera at 1:1000 dilution. Secondary Abs were goat anti-mouse, goat anti-rat, and goat anti-rabbit IgG conjugated to Alexa 488 or Alexa 568 (1:400; Molecular Probes). DAPI and Toto-3 (Molecular Probes) were used to stain DNA.

Detection of Apoptosis

We used an MEBSTAIN Apoptosis Kit Direct (MBL) to detect cell death in testes. Testes were dissected and fixed in 4% formaldehyde in PBX as described above. Fixed testes were washed in PBX, and cell death was detected according to the manufacturer's instructions.

Supplemental Data

Supplemental Data including genetic interactions of *Rap1* and *Rap2l* with *Gef26*, Rap1-GFP expression in the testis, and a proposed model of Gef26 function in regulating GSC anchorage are available at http://www.developmentalcell.com/cgi/content/full/10/1/117/DC1/.

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